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Similar Neutrophil-Driven Inflammatory and Antibacterial Responses in Elderly Patients with Symptomatic and Asymptomatic Bacteriuria

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Differential diagnosis of asymptomatic bacteriuria (ASB) and urinary tract infection (UTI) is based on the presence of diverse symptoms, including fever (≥38.5°C), rigors, malaise, lethargy, flank pain, hematuria, suprapubic discomfort, dysuria, and urgent or frequent urination. There is consensus in the medical community that ASB warrants antibiotic treatment only for patients undergoing urological procedures that lead to mucosal bleeding, catheterized individuals whose ASB persists for more than 48 h after catheter removal, and pregnant women. Pyuria is associated with UTI and implicates host immune responses via release of antibacterial effectors and phagocytosis of pathogens by neutrophils. Such responses are not sufficiently described for ASB. Metaproteomic methods were used here to identify the pathogens and evaluate molecular evidence of distinct immune responses in cases of ASB compared to UTI in elderly patients who were hospitalized upon injury. Neutrophil-driven inflammatory responses to invading bacteria were not discernible in most patients diagnosed with ASB compared to those with UTI. In contrast, proteomic urine analysis for trauma patients with no evidence of bacteriuria, including those who suffered mucosal injuries via urethral catheterization, rarely showed evidence of neutrophil infiltration. The same enzymes contributing to the synthesis of leukotrienes LTB_4 and LTC_4, mediators of inflammation and pain, were found in the UTI and ASB cohorts. These data support the notion that the pathways mediating inflammation and pain in most elderly patients with ASB are not quantitatively different from those seen in most elderly patients with UTI and warrant larger clinical studies to assess whether a common antibiotic treatment strategy for elderly ASB and UTI patients is justified.

Uncomplicated urinary tract infections (UTI) affect the health of approximately 150 million people worldwide and 7 million people in the United States annually; the majority of those affected are women (1). Ascending to the kidneys, UTIs lead to pyelonephritis, which is estimated to have an incidence of 12 cases per 10,000 women in the United States (2). Catheter-associated urinary tract infection (CAUTI), in particular, that seen following long-term catheterization, is characterized by biofilm formation on luminal and external urethral catheter surfaces and frequently causes nosocomial infections. CAUTIs are complicated UTIs because the rate of recurrence is higher and the effectiveness of short-term antibiotic treatment is decreased (3, 4). The incidence of CAUTIs, especially of CAUTIs contracted by elderly patients in long-term care centers and hospitals and by patients with spinal cord injuries and neurogenic bladders (3, 4), is estimated to be 0.9 to 1.5 million cases per year in the United States (1, 3). The infections are deemed potentially preventable complications of hospitalization by the Center of Medicare and Medicaid Services, and the associated costs are no longer reimbursed (3). *Escherichia coli* causes more than 70% of all community-acquired UTIs, while other bacterial pathogens contribute more frequently to UTIs in hospitalized patients (5). Among such pathogens are *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Streptococcus agalactiae*, *Staphylococcus saprophyticus*, *Klebsiella* spp., *Citrobacter* spp., *Enterococcus* spp., and fungal *Candida* species (5, 6). Treatment regimens for UTI differ, but a course of antibiotics over 1 to 3 days using trimethoprim-sulfamethoxazole, a β-lactam, a fluoroquinolone, or nitrofurantoin is typically prescribed (1). CAUTIs are often treated with a course of antibiotics over 5 to 7 days (3). The choice of antibiotic depends on the infectious agents and outcomes of urine specimen-specific antibiotic susceptibility tests. It is not uncommon that CAUTIs in patients with long-term catheterization are polymicrobial infections. The pathogens can benefit from a community lifestyle on catheter surfaces, sharing nutrient resources and jointly manipulating host defenses to avoid antibacterial peptides and phagocytosis (6, 7).

To diagnose UTI and CAUTI, the results from urine microscopy and urine culture (UC), dipstick tests, and clinical symptoms are considered. The symptoms include fever (≥38.5°C), rigors, altered mental status, malaise, lethargy, flank pain, costovertebral angle tenderness, hematuria, suprapubic discomfort, dysuria, and urgent/frequent urination. In patients with spinal cord injuries, increased spasticity and autonomic dysreflexia are also considered (3, 8). Most laboratories define UTI and CAUTI as the presence of 10^5 CFU/ml urine with UTI symptoms, but a threshold of 10^3 CFU/ml is also applied (3, 4). The distinguishing diagnostic features of UTI and CAUTI, compared to asymptomatic bacteriuria and catheter-associated asymptomatic bacteriuria (ASB and CAASB, respectively), are the patient's symptoms. The distinction is important because the medical community generally recom-

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mends treating UTI/CAUTI, but not ASB/CAASB, with antibiotics (3, 9, 10). If ASB and CAASB persist for longer than 48 h, guidelines for antibiotic treatment are less clear and risk of UTI may increase, thus justifying antibiotic treatment. To define ASB in women, $\geq 10^5$ CFU/ml urine in two consecutive clean-catch specimens and identification (ID) of at least one microbial species not considered to be a commensal organism of the vagina and perineum, such as *Gardnerella*, *Lactobacillus*, and *Corynebacterium* species, were previously suggested (11). Lowering the count to 10^4 CFU/ml urine for species considered less pathogenic, e.g., *S. agalactiae* and *Candida* species (11), and to 10^2 CFU/ml for catheterized urine specimens (8) is accepted practice in some clinical laboratories. A recent CDC report recommended defining CAUTI and CAASB as conditions requiring insertion and maintenance of a catheter for more than 2 calendar days prior to diagnosis (12). With the advent of advanced non-culture-based microbial identification procedures such as metagenomics (13–15) and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (16, 17), there is renewed interest as to which microbes should be considered commensals and opportunistic pathogens. Commensal organisms of the vaginal tract, the urinary introitus, and the perineum are often detected together with squamous epithelial cells in clean-catch urine samples (9).

ASB rarely affects young healthy adults, but its prevalence is higher in the presence of genitourinary abnormalities in pregnant, postmenopausal, and diabetic women (2% to 10%, 3% to 9%, and 9% to 27%, respectively), in elderly men and women living in long-term-care facilities, and in patients with spinal cord injuries who use catheters (more than 20% higher in both cases) (9). Antibiotic treatment of ASB is indicated during pregnancy due to the risk of birth complications and occasionally for hemodialysis and kidney transplant patients (9, 11). Pyuria is defined as the microscopically assessed presence of ≥ 10 leukocytes/mm^3 in uncentrifuged voided midstream urine and is not considered a diagnostic criterion of ASB (3, 9). It was reported to occur in 30% to 75% of patients catheterized for a short period (9), concurring with the notion that only some ASB cases constitute transient, self-limited bladder colonization (18). It is a conundrum that ASB may cause inflammatory responses leading to urothelial tissue injury while not causing symptoms. There is also no evidence that the causative agents are less virulent than in cases of UTI. Human immune responses in the urinary tract include resistance to microbial adhesion to urothelial surfaces, inflammatory signaling events mediated by TLR receptors, and the release of antibacterial peptides, complement factors, and immunoglobulins destined to opsonize and kill the pathogens. Toll-like receptor (TLR) engagement is followed by recruitment of phagocytic immune cells, particularly neutrophils, to the sites of the insult. Wound repair processes are also initiated (19, 20). These immune system activities also pertain to the defense against urethral catheter-associated microbial biofilms (21). Acute inflammation implicates the sensation of pain and development of fever, and prostanoids are among the main pain and fever signaling molecules (22, 23). The issue arises as to whether specific pathways contributing to pain and fever are disengaged in cases of ASB with pyuria compared to UTI.

Cells of the innate immune system and the urothelium are released into urine, and UTI-causing microbial agents are released into urine sediments. This can be measured by proteomic methods (13). We used that approach here to examine antimicrobial and inflammatory responses in 55 elderly patients hospitalized with trauma, comparing cases of ASB and UTI and of absence of bacteriuria (NoB). We assessed biological pathways contributing to UTI symptoms such as pain, fever, and overactive bladder quantitatively and interrogate current medical practice, which is the abstention from antibiotic treatment of elderly patients in most cases of ASB.

MATERIALS AND METHODS

Human subjects and urine specimens. A prospective, observational trial involving 129 elderly patients (≥65 years old) admitted to the hospital as a result of injury and consenting to participate in the study was performed between April and September 2013. The Internal Review Boards of the Mayo Clinic and the J. Craig Venter Institute (JCVI) approved the human subject protocol and the consent forms in application no. 12-010185 (24). All patients had a blunt mechanism of injury, with falls from a standing height in 76% of the cases. Urine samples were collected at admission within 30 min of catheterization (in cases in which catheterization was necessary), 72 h after admission into the hospital, and (in a few cases) up to 2 weeks after antibiotic treatment. To avoid contamination of urine, the catheters were closed sterile systems, and urine specimens were collected from the sample port above the catheter bag, which was swabbed clean with alcohol by practitioners wearing gloves. The specimens from six patients were clean-catch urine specimens. Patients with urogenital injuries were excluded from the study because such injuries might have confounded diagnosis of and immunological responses to UTI and ASB. A 2-ml urine aliquot was taken for standard urinalysis (UA) and UC tests. UA, Gram stain analyses, microscopy, and urine cultures were performed for most specimens. The clinicians were blinded to the results. Patient data such as age, sex, injury severity score (ISS), presence or absence of diabetes, urine glucose and protein levels, pre- and posttrauma antibiotic intake, and date of catheter insertion and length of time of catheterization were collected. All therapy, including antibiotic treatment, was based on the discretion of the treating clinician. Delayed consent was obtained after specimen collection; specimens were destroyed for those patients who refused consent.

Urine analysis. UA included dipstick measurements of nitrite concentrations (concentration) (≥1+), leukocyte esterase (LE) activity (≥1+), and hemoglobin, glucose, and total protein levels. Microscopic evaluations were performed to determine the abundance of leukocytes, erythrocytes (ERYs), epithelial cells, microorganisms, casts, and salt crystals. ASB was defined as $\geq 10^3$ CFU/ml of no more than two genera/species of microorganisms in a urinary catheter specimen or $\geq 10^5$ CFU/ml in a midstream voided specimen in the absence of UTI symptoms (3, 9). UTI was defined as the condition of a symptomatic patient with $\geq 10^3$ CFU/ml of no more than two species of microorganisms in the presence or absence of a urinary catheter within 48 h prior to sample collection. Patients catheterized upon admission whose catheters were in place for at least three consecutive days and who developed UTI or ASB were considered to have contracted CAUTI or CAASB. Pyuria was defined as ≥4 neutrophils per high-power field (HPF) and microscopic hematuria was defined as ≥3 red blood cells (RBC) per HPF. The mean costs of UTI treatment were calculated based on Centers for Disease Control estimates of $862 to $1,007. UTI symptoms included fever (≥38.5°C), rigors, altered mental status, malaise, lethargy, flank pain, costovertebral angle tenderness, hematuria, suprapubic discomfort, dysuria, and urgent/frequent urination. In patients with spinal cord injuries, increased spasticity, autonomic dysreflexia, and unease were also included.

Shotgun proteomics. Urine specimens of a 20-to-50-ml volume not used for UA or UC were centrifuged at 5,000 × *g* at 4°C for 15 min. The urine sediment fraction, also termed the urinary pellet (UP), was resuspended with circa 10 ml phosphate-buffered saline (PBS), centrifuged, and resuspended in 200 to 400 μl PBS before storage at −80°C until further use. Aliquots of the UP samples were thawed and subjected to denaturation and lysis using a solution containing 8 M urea, 50 mM dithiothreitol (DTT), 5 mM EDTA, and 1% SDS (vol/vol) and sonication

followed by filter-aided sample preparation (FASP) in Vivacon filter devices with a 10-molecular-weight cutoff (MWCO) (Sartorius AG, Germany) as previously reported (25). Quantities of a lysate with approximately 10 to 100 μg total urinary protein (estimated from Coomassie brilliant blue-G250 staining intensities of protein bands in SDS-PAGE gels) were digested twice with trypsin (25). SDS-PAGE was used for protein quantitative estimates for two reasons. First, urine and urine sediment (UP) contain chemical compounds that interfere with colorimetric protein concentration measurements. Second, UP samples have significant quantities of insoluble protein, leading to underestimation of protein content when colorimetric assays are used. The filtrate of the FASP process, enriched for peptides, was desalted using the StageTip method (26), and approximately one-fifth of the desalted filtrate was analyzed by liquid chromatography-tandem MS (LC-MS/MS) using a previously reported method (25). Briefly, an Ultimate 3000-nano LC workstation and a Q-Exactive mass spectrometer system coupled with a FLEX nano-electrospray ion source (all components from Thermo Scientific, USA) were used. Peptides were separated on a PicoFrit C_{18} analytical column (BetaBasic; New Objective, Inc., Woburn, MA) (75 μm by 10 cm, 5 μm particle size, 150 Å) at a flow rate of 300 nl/min. A 130-min LC gradient starting with 98% solvent A (0.1% formic acid–water) and increasing to 35% solvent B (0.1% formic acid–acetonitrile) over 110 min and 80% solvent B over 15 min was employed. Eluting peptides were sprayed at a voltage of 2.0 kV and acquired in a MS-data-dependent mode using XCalibur software (version 2.2; Thermo Scientific). Survey scans were acquired at a resolution of 70,000 (m/Δm) over a mass range of *m/z* 250 to *m/z* 1,800 with an automatic gain control (AGC) target of 10^6. For each cycle, the 10 most intense ions were subjected to fragmentation by high-energy collisional dissociation, with a normalized collision energy level of 27%. Peptide ion fragments from the MS/MS scans were acquired at a resolution of 17,500 with an AGC target of 5×10^4 ions. Dynamic exclusion was enabled, with MS/MS ion scans repeated once for 20 s and then excluded from further analysis. Unassigned ions and those with a charge of +1 were excluded from further MS/MS analysis. Peptide mixtures derived from UP samples were usually run twice on the LC-MS/MS system.

Database searches and quantitative proteomic analysis. Raw MS files were processed using the Sequest HT search engine integrated in the Proteome Discoverer platform (version 1.4; Thermo Scientific). We modified our use of the complete human UniProtKB database (Release 2013_6; 88,295 human sequences) by employing a 75% sequence identity threshold to reduce protein redundancy and an application in the CD-HIT software suite (27). The human proteome database subset contained 27,151 protein sequences. To this database, we added protein sequences derived from 21 microbial genomes corresponding to the species accounting for approximately 98% of all of the ASB and UTI cases under study (28). The final database consisted of 97,919 protein sequences from 22 organisms (see Table S1 in the supplemental material) (29). False-discovery rates (FDR) of protein IDs were determined by searching a reversed version of the database (30) and accepting IDs with an FDR value set at 1%. Replicates of raw MS files were combined during the database search, thus generating a single msf file for each urine sample. Identifications of bacteria required the presence of at least two proteins for a given species with at least two unique peptides as determined by Proteome Discoverer search results. If a bacterial species was identified with the highest protein ID number, criteria for second and third species from the same phylogenetic family were more stringent due to a large number of nondifferentiable peptides and missing protein sequence entries in their genomes, a problem which we have elaborated on recently (29). Therefore, we were cautious regarding the identification of species from phylogenetically similar groups, such as the five species in the family of *Enterobacteriaceae* and two species in the *Staphylococcus* genus.

Quantitative analysis with the MaxQuant software application. The raw MS files were imported into the MaxQuant software suite (version 1.4.2), accepting the default settings for MS^1 peak integration-based protein quantification. Human protein ID data obtained with the Andromeda search engine were selected followed by label-free quantification (LFQ) (31). LFQ analysis quantifies and normalizes integrated MS^1 peak areas from high-resolution MS data across many protein datasets. In addition, the data were processed by the use of the intensity-based absolute protein quantity (iBAQ) method and by adjusting protein quantities with the total protein analysis (TPA) method (29). The iBAQ analysis sums the raw integrated MS^1 peak intensities of all peptide ion observations for a given protein divided by the number of theoretically observable peptides, which are calculated by *in silico* digestion of protein sequences, including fully tryptic peptides with a length of 6 to 30 amino acids (32). The TPA method normalizes the peak intensity value of protein i (LFQ_i) by dividing it by the sum of all LFQ intensities of the measured proteome; each $LFQ_i/\Sigma LFQ$ quotient is then divided by the number of theoretical tryptic peptides per protein, yielding the TPA_i value. This analysis revealed that the human proteome had a dynamic abundance range spanning over 5 orders of magnitude, allowing quantification of urinary proteins in the range of approximately 1 pM to 10 nM. Only proteins identified with at least two unique peptides were used for quantification.

Data normalization and statistical analysis. We selected a subset of 69 entries from the 100 MaxQuant proteomic datasets for in-depth statistical analysis, with the goal being to interpret pathobiological differences. The selections were based on sufficient depth of proteomic data (at least 300 protein IDs), elimination of the greater part of the post-antibiotic-treatment datasets, and good correlation of UC and proteomic data with respect to the identification of bacterial pathogens (see Dataset S1 in the supplemental material). Agreement of UC and proteomic data at the microbial genus level was observed in two-thirds of the cases. The definitions of clinical groups were as follows: for UTI, (i) the presence of microbial pathogens identified by both UC and proteomics, only by proteomics, or only by UC in cases of Gram-positive bacterial species, which can be missed by proteomic analysis due to insufficient lysis, and (ii) the presence of UTI symptoms; for ASB, (i) the presence of microbial pathogens detected as described for UTI and (ii) the absence of UTI symptoms; and for the absence of bacteriuria (NoB), (i) no reliable identification of a bacterium and (ii) the absence of UTI symptoms.

iBAQ values were used to calculate scores corresponding to the summed intensity values of the proteins relevant to specific functional roles and cell-specific expression: the neutrophil activation and degranulation (NAD) score, the erythrocyte (ERY) score, and the complement activation and acute-phase-response (CAP) score. Protein assignments to determine NAD, ERY, and CAP scores are listed in Table S2 in the supplemental material. The sum of iBAQ values for a distinct protein subset divided by the sum of all iBAQ values yielded the score for a UP sample. Statistical and clustering analyses were performed from LFQ-quantified proteome datasets. First, the human proteins (3,492 entries) represented in LFQ datasets were filtered to reduce noise linked to low-abundance proteins with few peptide-spectrum matches and often present in only a few UP samples. These proteins cannot be reliably quantified and introduce noise in statistical analyses. One filter required a protein to have an LFQ abundance of less than 4 orders of magnitude lower than the most abundant protein (averaged from all 69 datasets). Then, 105 proteins with a median abundance value of 0 across all datasets were eliminated, leaving 800 protein entries in the filtered dataset. Imputations for missing protein values were performed to enable principal component analysis (PCA) in the Perseus application (33). PCA requires complete datasets and reduces the dimensionality of the data to identify the directions along which the variations among the proteomic datasets of UP samples are maximal.

Filtered LFQ datasets were used to determine statistically significant protein differences revealed by comparisons of the three clinical groups, and the derived data are described in Results. Following this, we considered additional options for data normalization to ensure that the biological interpretation of differential LFQ abundance data was justified given that normalization of "omics" data from clinical samples with high inherent variability such as urine is challenging. Normalizing based on the fractional quantity of the human proteome versus the total proteome in a

TABLE 1 Human subjects and overview of cases of UTI and ASB and their microbial causes

Patient group	No. of patients (no. of samples)[b]	No. of patients[c]			No. of patients of indicated sex		No. of cases (no. of samples)[d]						
		Cath. (Adm)	Cath. ≥ 3 days	Cath-ass. bacteriuria	Female	Male	NAD ≥ 15%	ERY > 6%	Protein (Ad)	Gram (−)	Gram (+)	Abx 72 h	PAP bacteriuria
ASB	26 (29)	20	11	14	12	10	18	7	8	20 (9)	8 (4)	14	3
UTI	15 (16)	12	5	5	9	6	13	7	10	12 (3)	9 (3)	11	
NoB_{Ad}[a]	13 (13)	12					3	6					
NoB	12 (14)	11	5		6	6	4	6	4				

[a] NoB_{Ad}, no bacteriuria (at admission).
[b] Some patients contributed two samples for analysis.
[c] Cath. (Adm), catheter insertion at admission; Cath. ≥ 3 days, catheterization for >2 calendar days; Cath-ass. bacteriuria, catheter-associated bacteriuria.
[d] NAD ≥ 15%, proteomic neutrophil activation and degranulation (NAD) score greater than 15%; ERY > 6%, erythrocyte (ERY) score greater than 6%; Protein (Ad), greater than 250 mg protein/24-h voiding at admission; Gram (−), Gram-negative bacteria identified; Gram (+), Gram-positive bacteria identified; Abx 72 h, antibiotic treatment 72 h after admission; PAP bacteriuria, bacteriuria after antibiotic treatment.

dataset (bacterial peptides were also identified) was not required because the LFQ method does not compute MS^1 integrated peak areas for peptides assigned to a specific protein by integrating correction factors based on the total peak area LFQ quantity in a LC-MS/MS experiment. Going a step back, on the basis of the aforementioned rationale, we also did not normalize for quantitative differences in peptide loading for a LC-MS/MS experiment.

Next, we normalized based on the fact that proteins have different M_r values and generate different numbers of tryptic peptides. The TPA method includes this normalization step, thus ensuring that the quantities of proteins with low M_r values are not underestimated. A second normalization step was performed in light of the fact that UP sample lysates had different total protein quantities, and only a fraction of the lysate was subjected to the FASP process in more than 50% of the cases. We thus calculated a FASP loading correction factor by which the TPA values for all proteins identified in a given UP sample were multiplied. These factors are listed in Dataset S1 in the supplemental material and ranged from 1 to 9.1. The correction factor-adjusted TPA datasets were then subjected to the same statistical analyses also applied to LFQ datasets. The average quantitative estimates of protein in urinary pellets for the UTI and ASB samples were similar, 205 μg/ml and 199 μg/ml, respectively, but the estimate was lower for NoB samples (123 μg/ml). Thus, the normalization process was expected to influence statistical analyses that included NoB datasets as a group more than those comparing ASB and UTI datasets.

The Multiple Experiment Viewer (MeV) software tool (34) was used to perform hierarchical clustering (HCL) analysis using the Pearson correlation metric, accepting the parameters of absolute distance and complete linkage analysis. To identify statistically significant protein differences in the comparisons of UTI, ASB, and NoB groups, nonparametric unpaired Wilcoxon rank sum and unequal-variance *t* tests were performed. The unequal-variance *t* test has been described as performing at least as well as the Wilcoxon rank sum test when the goal is to compare two populations based on samples of unrelated data (35). Functional roles of proteins linked to immunity and inflammation in response to insults and injury in the urinary tract were deduced from information in the literature and the UniProt database (36).

RESULTS

Study purpose and design. As part of an observational trial of 129 elderly patients (≥65 years old) admitted to the hospital following accidental injury, most of whom were catheterized on admission, urine sediments were examined to determine whether undiagnosed UTI and ASB potentially contributed as causes of the trauma suffered. We previously reported that 25% of the cases examined by urinalysis and urine culture showed evidence of bacteriuria on admission (24), suggesting that the rationale to declare CAUTI a preventable disease in the nosocomial environment is confounded by preexisting bacteriuria in elderly patients (8). Here, we analyzed the metaproteome of 100 urinary sediment samples from all patients with evidence of bacteriuria and 12 patients with negative UC test results. The total number of patients was 55, 43 of whom were catheterized on admission. The intent was to understand patterns of innate immunity and inflammation in cohorts diagnosed with ASB and UTI. Twelve cases defined by the absence of bacteriuria (NoB) were included to ensure that the effect size and extent of measurable immune responses were sufficient for analysis. Proteomic data were examined for 54 admission samples, 37 samples collected at the 72-h time point, and 9 samples collected up to 2 weeks after antibiotic treatment had been administered. The patients did not have clinical evidence of febrile kidney infections prior to or during their hospital stays.

Bacteriuria in elderly patients and associated clinical data. The microbial agents identified by UC or by proteomics or by both methods were *E. coli* (18 cases), *Enterococcus* (7 cases), *Klebsiella* (5 cases), *Staphylococcus aureus* and *P. aeruginosa* (3 cases each), *Citrobacter*, *P. mirabilis*, and *S. agalactiae* (2 cases each), and *Aerococcus*, *Enterobacter*, *Morganella*, and *Candida* (1 case each). Details are provided in Dataset S1 in the supplemental material. For all microbial genera/species identified more than once, at least one case was reported to be a UTI, and at least one case was reported to be ASB. Based on the length of time of catheterization prior to diagnosis, 5 of 16 UTI cases were CAUTIs and 14 of 29 ASB cases were catheter associated (CAASB) (Table 1). Female patients were slightly overrepresented compared to male patients in both the UTI and ASB groups. UC data indicated that 12 UTI and 18 ASB specimens had colony counts of more than 10^5 per ml. The colony count numbers were 4 and 10 for the 10^4 to 10^5 CFU/ml range, respectively. Six UTI patients, 8 ASB patients, and 4 NoB patients had an injury severity score (ISS) equal to or greater than 5. There was no evidence that severe injury correlated with lacking UTI symptoms (ASB), for example, due to a higher dose of analgesics taken in by these patients. All but nine proteomic analyses pertained to the first 3 days of the hospital stay, and the earliest day of antibiotic treatment was day 3. Therefore, bias associated with a long hospital stay and with its effect on UTI versus ASB diagnosis was not introduced. The average ages of patients in the UTI, ASB, and NoB diagnosis groups were 82, 83, and 85 years, respectively. Age was not a variable distinguishing the groups. Seven individuals were diabetic, a disease increasing susceptibility to infections and kidney function decline. Such cases were represented in all

three groups. While certain pathogens (e.g., *S. aureus*, *K. pneumoniae*, and *E. faecalis*) cause increased rates of bacteriuria in nosocomial environments, there is no evidence that the elicited immune responses in patients were fundamentally different in the hospital setting versus the community setting. Therefore, separating analyses with respect to the admission samples and 72-h time point UP samples to distinguish ASB from UTI was not justified.

Bacteriuria and antibiotic treatment. In 68% of the cases of bacteriuria, UC and proteomic data were in agreement on pathogen identifications. UC data reported bacterial IDs more frequently than proteomic data (see Dataset S1 in the supplemental material), perhaps due to reduced sensitivity of detection of bacterial proteins in a high-level host proteomic background or to ineffective bacterial cell lysis prior to proteomic analysis. On the other hand, erroneous IDs of microbial pathogens by UC, e.g., via growth of contaminants in culture, could not be ruled out. Two or more pathogens were identified in five patients. A low prevalence of polybacterial cases was expected because nearly all catheter-associated specimens reflected short-term catheterization. For example, nearly 700 bacterial proteins of *K. pneumoniae*, *Gardnerella vaginalis*, and *Aerococcus urinae* were identified for urine specimen 168_a. Increased prevalences of distinct bacteria in any of four categories (ASB, CAASB, UTI, and CAUTI) were not evident, supporting the notion that short-term catheterization does not allow biofilm-adapted species such as *P. mirabilis* and *P. aeruginosa* to outcompete bacteria with a preference for a planktonic lifestyle. Patients were treated with antibiotic drugs on a case-by-case basis. In three instances of CAASB, caused by *K. pneumoniae*, *Enterococcus* sp., or *Enterobacter* sp., bacteriuria persisted after antibiotic treatment. Four, seven, and four patients diagnosed with UTI, ASB, and NoB, respectively, had knowingly received antibiotic treatment in the 3 weeks prior to the traumatic event (see Dataset S1). There is no evidence that this antibiotic treatment introduced bias as it pertains to the comparative analysis of immune responses in cases of UTI versus ASB.

Profiling immune responses to pathogens in the urinary tract. A metaproteomic approach applied to bacteriuria in spinal cord-injured patients (13) was further developed to characterize innate immune responses in cases of UTI quantitatively (29). The observation of pyuria (leukocyte counts of ≥4 or 10 per HPF in urine) implicates the infiltration of activated neutrophils as the main drivers of the acute immune response in UTIs (19). Urothelial cells and macrophages contribute to the release of proinflammatory and antibacterial molecules and amplify the host defense in the urinary tract (20, 37). It has not been investigated in studies on clinical samples, to our knowledge, whether these immune responses involve a range of neutrophil effectors to similar degrees in cases of UTI caused by different pathogens and whether the responses functionally and quantitatively differ in patients diagnosed with ASB. One report has suggested that only 25% of the ASB cases in women aged 18 to 49 years showed neutrophils in urine on the basis of dipstick tests (38). In contrast, pyuria was reported to be more prevalent in catheterized and elderly patients without UTI symptoms. Using pyuria as a diagnostic criterion to distinguish UTI from ASB has been discouraged (3, 9). Individuals who are catheterized over a long period are desensitized and may fail to experience typical UTI symptoms. In this report, we address the knowledge gap on immune responses in instances of diagnoses of ASB versus UTI in a cohort of elderly patients with short-term catheterization.

Strong evidence for the presence of activated neutrophils in the urinary tract during ASB. First, we analyzed the urinary proteomes quantified with the LFQ method representing 26 cases of ASB and 27 cases of NoB, followed by statistical evaluation of the data. Using the unequal-variance *t* test, the results for 94 proteins had a *P* value of ≤0.01; among those 94 proteins, 37 were increased in abundance in the ASB group. Strikingly, 29 of the 37 proteins are enriched in and functionally associated with activated neutrophils. Most of the remaining proteins that were increased in abundance in the ASB group participate in immune cell signaling pathways (tyrosine-protein phosphatase PTPN6) or maturation, repair, and exocytosis of membrane vesicles (dysferlin, reticulon-3, and actinin α-1). While not selectively associated with neutrophils, such functions are clearly important in neutrophils for their ability to migrate (39), degranulate, and act as phagocytes during infection (40). Information on the proteins' functional roles, abundance differences, and localizations in granules and extracellular traps of neutrophils is supplied in Table 2 and Table 3. The names of all the differentially abundant proteins and the supporting statistical data are provided in Dataset S2 in the supplemental material.

Antimicrobial and proinflammatory proteins secreted from the neutrophil cytosol into the extracellular space or phagolysosomes were significantly more abundant in the ASB dataset than in the NoB dataset (Table 2). While enzymes contributing to leukotriene B4 (LTB_4) synthesis (ALOX5, ALOX5AP, and LTA4H) were increased in abundance, a key enzyme for prostaglandin H2 biosynthesis from arachidonic acid (COX2) and γ-glutamyltranspeptidase 1 (GGT1), which metabolizes LTB_4 via LTC_4 to LTD_4, were decreased in abundance in the ASB group (Fig. 1). The enzyme catalyzing the ω-oxidation of LTB_4, LTB_4 omega-hydroxylase 2 (CYP4F2), while of relatively low abundance compared to other proteins contributing to leukotriene metabolism, was also increased in abundance in the ASB group compared to the NoB group. In contrast, four enzymes catalyzing mitochondrial β-oxidation of 20-carboxyl-LTB_4, namely, the acyl-CoA oxidase ACADM, the CoA hydratase ECHM, the hydroxyacyl-CoA dehydrogenase HCDH, and the 3-oxoacyl-CoA thiolase ACAA2, were strongly decreased in abundance in the ASB group versus the NoB group (Fig. 1). Synthetic and metabolic pathways for LTB_4 were characterized before (43) but not in the context of UTI and ASB. Our data are consistent with a higher concentration of LTB_4 and LTC_4 in the activated neutrophils which had previously infiltrated the urinary tract upon recognition of an invading pathogen and a decrease in leukotriene catabolism. Neutrophil proteins implicated in chemotaxis, extravasation, and trafficking and formation of secondary granules were also increased in abundance in the ASB group (Table 3). A Wilcoxon rank sum test (WRST), with a *P* value of ≤0.05 with multiple-testing correction (see Dataset S3 in the supplemental material), revealed high statistical significance of changes for nearly all of the proteins referred to above. This test identified additional neutrophil proteins, such as the antimicrobial proteins defensin-1, cathelicidin, and bactericidal permeability-increasing protein, with quantitative increases in the ASB group versus the NoB group. These peptides are present in the azurophilic granules of neutrophils. The results were similar for two inflammatory chemokines, S100A8 and S100A12, both of which are abundant in the neutrophil cytosol, and for protein subunits of the NADPH oxidase complex, a phagolysosomal enzyme generating reactive oxygen species (ROS) to kill the engulfed

TABLE 2 Neutrophil proteins associated with antimicrobial functions, including proteolysis, iron chelation, membrane disruption, and reactive oxygen generation, significantly changed in abundance in the comparison of ASB to NoB[a]

Protein name	Short name	ASB mean	NoB mean	ASB/NoB	*P* value[b]	Azur Gr[c]	Spe/G Gr[d]	NET[e]
Myeloperoxidase	MPO	5,165	1,133	4.56	0.0002	x	x	x
Myeloblastin	PRTN3	2,221	523	4.25	0.0003	x	x	x
Cathepsin G	CTSG	3,783	1,138	3.32	0.0006	x	x	x
Lactotransferrin	LTF	9,321	3,378	2.76	0.0019	x	x	x
Neutrophil elastase	ELANE	2,769	1,095	2.53	0.0026	x	x	x
Neutrophil gelatinase-associated lipocalin	LCN2	745	203	3.66	0.0025	x	x	
Bactericidal permeability-increasing protein	BPI	613	173	3.53	0.0027	x	x	
Azurocidin	AZU1	2,170	737	2.95	0.0027	x	x	x
Resistin	RETN	86	24	3.62	0.0041	x	x	x
Eosinophil cationic protein	RNASE3	311	112	2.78	0.0079	x		
Cytochrome *b*-245 heavy chain	CYBB	312	74	4.24	0.0002			
Cytochrome *b*-245 light chain	CYBA	305	71	4.30	0.0017			
Lysosome-associated membrane glycoprotein 2	LAMP2	101	29	3.50	0.0006	x	x	
Arachidonate 5-lipoxygenase-activating protein	ALOX5AP	92	24	3.78	0.0002			
Arachidonate 5-lipoxygenase	ALOX5	51	18	2.84	0.0005			
Leukotriene A-4 hydrolase	LTA4H	176	85	2.06	0.0096		x	
Gamma-glutamyltranspeptidase 1*	GGT1	66	200	0.33	0.0010			
Cytochrome *c* oxidase subunit 2*	COX2	65	234	0.28	0.0066			

[a] Designations marked with an asterisk represent proteins not characterized in neutrophils. ASB mean, arithmetic mean of LFQ values from 26 datasets representing asymptomatic bacteriuria; NoB mean, arithmetic mean of LFQ values from 27 datasets representing no bacteriuria; ASB/NoB, ratio of means of protein abundance (ASB group versus NoB group); x, protein localized in indicated entity.
[b] The adjusted *P* values pertain to results of an unequal-variance *t* test.
[c] Azur Gr, presence of protein in azurophilic granules of neutrophils (41).
[d] Spe/G Gr, presence of protein in specific/gelatinous granules of neutrophils (41).
[e] NET, presence of proteins detected in extracellular neutrophil traps (42).

bacteria. Its membrane components CYBA and CYBB were differentially abundant in the *t* test experiment and are included in Table 2. By normalization based on the use of different protein loading quantities for each UP lysate in LC-MS/MS analyses, WRST tests were performed for proteomic datasets quantified by the TPA method with integration of a corrector factor (see Materials and Methods). Among the 45 proteins whose abundance was most significantly increased (in cases of ASB versus NoB), 20 proteins are highly expressed in neutrophils, and all 20 were also significantly increased in the LTQ-based analysis. In addition, quantitative increases for 10 of the 45 most abundant proteins, matched in the TPA- and LFQ-based analyses, pertained to the cytoskeleton (see Dataset S3). This finding strongly supports the notion that biological interpretations of quantitative differences comparing proteomic data from ASB and NoB cohorts are justified.

Neutrophil-driven immune responses in cases of UTI resemble those of ASB. In the equivalent unequal-variance *t* tests (*P* value at ≤0.02) comparing 16 UTI and 27 NoB datasets, 20 of the 23 proteins that were increased in abundance in the UTI group matched those seen in the ASB-versus-NoB comparison. Three additional proteins whose abundance was significantly changed in the UTI-versus-NoB comparison were the CD177 antigen, a neutrophil surface marker (44); histone H4, a nuclear protein with antimicrobial properties present in neutrophil extracellular traps (NET) (45); and calponin-2, an actin cytoskeleton protein regulating the motility and phagocytosis of leukocytes. The proteins were increased in abundance in the ASB-versus-NoB comparison, setting the *P* value threshold just a little higher, at 0.033. Of the enzymes involved in the biosynthesis of eicosanoids, LTA4H, ALOX5, and COX2 were changed in abundance at levels comparable to those seen in the results of the ASB-versus-NoB analysis. Among the proteins decreased in abundance in the ASB and UTI datasets with results showing statistical significance, compared to NoB datasets, there was agreement for 19 proteins, half of which contributed to mitochondrial energy metabolism (see Dataset S2 in the supplemental material). Clearly, the agreements observed for differentially abundant proteins (UTI versus NoB and ABS versus NoB) strongly support the notion that neutrophil infiltration and the activities of proteins expressed by these cells are shared features of the immune response to microbes invading the urinary tract in elderly patients diagnosed with ASB and UTI. There was no quantitative evidence for attenuated immune response pathways in the ASB cohort versus the UTI cohort, as further supported by results of the normalized proteomic analysis. Thirty-nine of the 45 proteins most significantly increased in abundance in the UTI dataset compared to the NoB dataset, using the TPA quantification method with a WRST, were present in the corresponding dataset where the LFQ-based method was used (see Dataset S3). There was also a lack of separate clusters for ASB and UTI cases in the plot shown in Fig. 2. Neutrophil activation, associated with high NAD scores, was observed for the large majority of UTI and ASB cases and indicates an acute immune response to infection. An NAD score of ≥30% correlated well with leukocyte counts of ≥31 cells/HPF. Bacterial cell counts of less than 10^5 per ml, depicted in the plot, revealed that lower cell counts did not correlate strongly with lower NAD scores or diagnosis of ASB.

Complement activity and vascular injury. Proteomic analysis of urine sediments allows inferences of complement activity, coagulation, and vascular injury (29). The summed abundances of proteins representing the three categories were plotted for the 69

TABLE 3 Neutrophil proteins associated with chemotaxis, extravasation, trafficking, and formation of secondary granules significantly changed in abundance in the comparison of ASB to NoB[a]

Protein name	Short name	ASB mean	NoB mean	ASB/NoB	*P* value[b]	Spe/G Gr[c]	C+ME[d]	NE+CD[e]	SV+N-DG[f]
Protein-arginine deiminase type 4	PADI4	126	35	3.57	0.0002	x		x	
Protein-arginine deiminase type 2	PADI2	49	11	4.28	0.0009			x	
Myeloid cell nuclear differentiation antigen	MNDA	764	345	2.21	0.0092	NET		x	
Unconventional myosin-If	MYO1F	34	11	3.05	0.0011				x
Reticulon-3*	RTN3	85	15	5.81	0.0014				x
Grancalcin	GCA	475	135	3.52	0.0026	x			x
Dysferlin*	DYSF	79	18	4.33	0.0031	x			x
Actinin alpha 1*	ACTN1	1,213	571	2.12	0.0048	x			x
Ras-related protein Rab-27A*	RAB27A	65	21	3.06	0.0055	x			x
Synaptic vesicle membrane protein VAT-1 homolog	VAT1	183	69	2.66	0.0071	x			x
Integrin alpha-M	ITGAM	383	125	3.06	0.0010	x	x		
Integrin beta-2	ITGB2	317	114	2.78	0.0048	x	x		
Matrix metalloproteinase-9	MMP9	1,099	265	4.15	0.0011	x	x		
Neutrophil collagenase	MMP8	185	51	3.60	0.0075	x	x		
Myeloid-associated differentiation marker	MYADM	50	7	6.88	0.0021		x		
Tyrosine-protein phosphatase no-receptor type 6*	PTPN6	343	116	2.96	0.0025	x	x		
Vasodilator-stimulated phosphoprotein	VASP	127	41	3.07	0.0037		x		
ADP-ribosyl cyclase 2	BST1	115	29	4.03	0.0037		x		

[a] Designations marked with an asterisk represent proteins not characterized in neutrophils. ASB mean, arithmetic mean of LFQ values from 26 datasets representing asymptomatic bacteriuria; NoB mean, arithmetic mean of LFQ values from 27 datasets representing no bacteriuria; ASB/NoB, ratio of means of protein abundance (ASB group versus NoB group); NET, proteins detected in extracellular neutrophil traps (42); x, protein localized in indicated entity.
[b] The adjusted *P* value pertains to results of an unequal-variance *t* test.
[c] Spe/G G, presence of protein in specific/gelatinous granules of neutrophils (41).
[d] C+ME, proteins associated with neutrophil chemotaxis, migration, and extravasation.
[e] NE+CD, proteins associated with nuclear envelope/chromatin decondensation in neutrophils.
[f] SV+N-DG, proteins associated with secreted vesicles and granule maturation or degranulation in neutrophils.

UTI, ASB, and NoB cases (Fig. 2). High erythrocyte protein content (ERY score) suggested vascular injury and leakage of these cells and their proteins into urine. High content of proteins implicated in the complement system and acute-phase responses (CAP score) suggested activity of these inflammatory pathways in the urinary tract. High CAP and ERY scores did not correlate well with evidence of strong neutrophil activation. NAD scores of greater than 20% were observed in most UTI and ASB cases and in a few NoB cases, e.g., UP samples 95_a, 95_b, and 41_a, an observation that also influenced the principal component analysis results (Fig. 3). Neutrophil infiltration into the urinary tract in the absence of identified microbial insults occurred. The majority of NoB cases (20 of 27 cases) were on the right side of the plot in Fig. 2, with NAD scores of lower than 20%. While vascular injury of the urinary tract is rare in normally voiding healthy individuals, 80% of the patients in the cohort under study were catheterized. Placement of catheters causes urothelial injury followed by hematuria, which is evident from the survey of high ERY scores for many cases displayed in Fig. 2. Consequently, hematuria is not useful as a diagnostic biomarker of UTI or ASB in catheterized elderly human subjects.

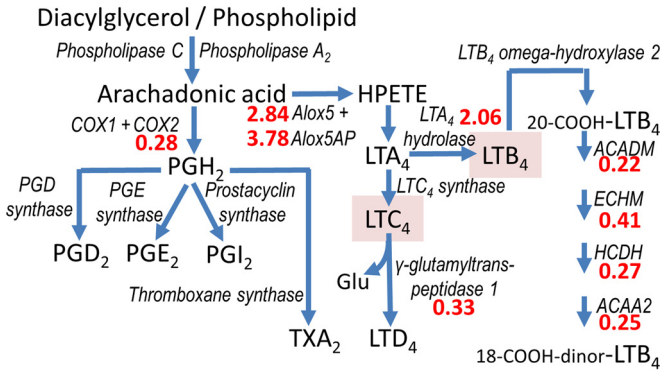

FIG 1 Changes in leukotriene metabolism for ASB cases compared to absence of bacteriuria. The schematic shows the pathways for eicosanoid biosynthesis and catabolism and relative changes in the abundance of specific enzymes, comparing ASB with NoB datasets. The pathway is highly active in activated neutrophils. Numbers greater than 1 reflect quantitative increases in the ASB datasets. Full names for some of the enzymes are provided in the text and Table 2. HPETE, 5-hydroperoxyeicosatetraenoic acid; PGD, prostaglandin D; PGE, prostaglandin E; TXA, thromboxane A.

High similarity of immune responses in cases of UTI and ASB. Unequal-variance *t* tests with a *P* value set at ≤0.02 comparing 16 and 26 datasets for UTI and ASB, respectively, resulted in the identification of only seven differentially abundant proteins (see Dataset S2 in the supplemental material). Only kininogen-1 (KNG1), angiopoietin-related protein 2 (ANGPTL2), and matrix-remodeling-associated protein 8 (MXRA8) showed abundance decreases for the comparisons of UTI versus ASB and UTI versus NoB, and the quotients for the latter comparison were smaller. The order of decrease allows the hypothesis that the ASB condition reflected an attenuated immune response. KNG1, a thiol protease inhibitor, has a variety of functions in vascular inflammation, adhesion, and coagulation, some of which are mediated by its truncated peptides, such as bradykinin. The protein is expressed in the kidneys and secreted into urine. There is only one published report on its functional role in the context of innate immunity. KNG-1 was associated with decreased expression in myeloblastin antibody-stimulated neutrophils compared to unstimulated neu-

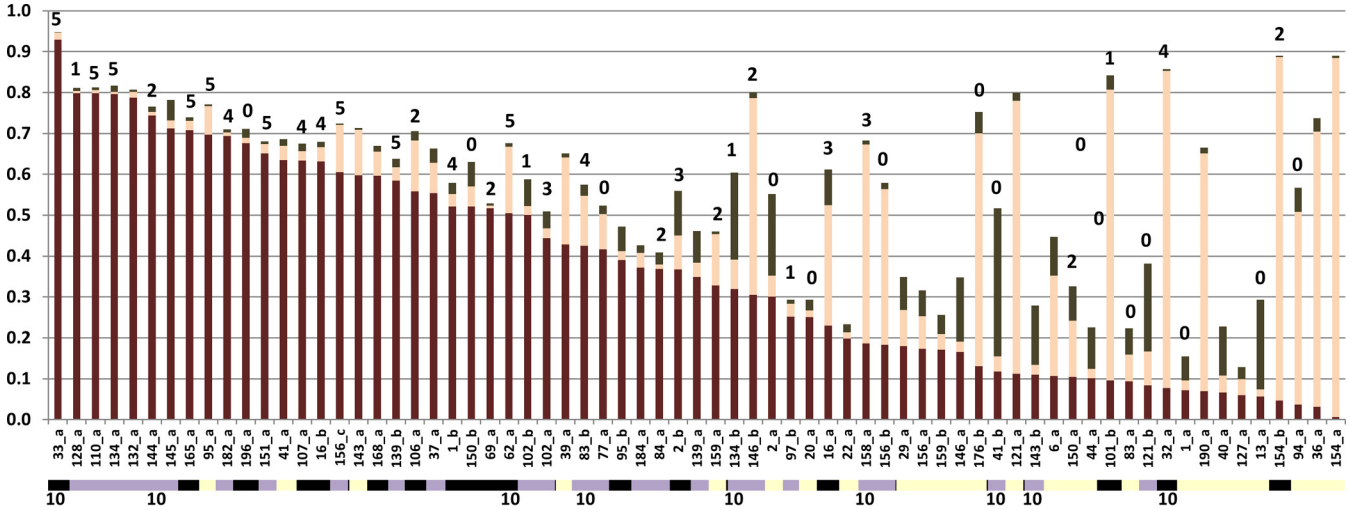

FIG 2 Quantitative representation of protein groups associated with activated neutrophils, erythrocytes, or the complement system and acute-phase responses. The plot has 69 proteomic profiles from urine specimens, with sample identifiers listed on the *x* axis. The bar segments represent a quantitative fraction of the proteome compared to the total proteome in the urine sediment (from a distinct patient sample), as assessed by the iBAQ method. The segments in each bar represent neutrophil activation and degranulation (NAD scores) (bottom; purple), erythrocyte proteins (ERY score) (middle; pink), and proteins participating in the complement system and acute-phase responses (CAP scores) (top; gray). Proteins included in the score calculations are defined in Table S2 in the supplemental material. The sample order, from left to right, starts with the highest NAD score and ends with the lowest. Above some of the bars, leukocyte counts from microscopy data are shown. The numbers represent counts per high-power field as follows: 5, >100 cells; 4, 51 to 100 cells; 3, 31 to 50 cells; 2, 11 to 30 cells; 1, 4 to 10 cells; 0, <3 cells. Below the *x* axis, the horizontal bar indicates diagnosis of UTI (black), ASB (lilac), or NoB (yellow) for each of the 69 samples. A value of 10 represents 10^4 to 10^5 CFU/ml. Other cases of bacteriuria had $>10^5$ CFU/ml.

trophils (60). ANGPTL2 has sequence similarity to angiopoietins, proteins influencing vascular functions, while MXRA8 belongs to a group of proteins modulating the extracellular matrix. There is no knowledge on the proteins' expression levels in urothelial cells, and they were of very low abundance in both the ASB and UTI proteomic profiles. MXRA8 had similar statistical evidence in a WRST, with a *P* value at 0.021 (UTI versus ASB), but this expression difference was not confirmed in the statistical analysis of nor-

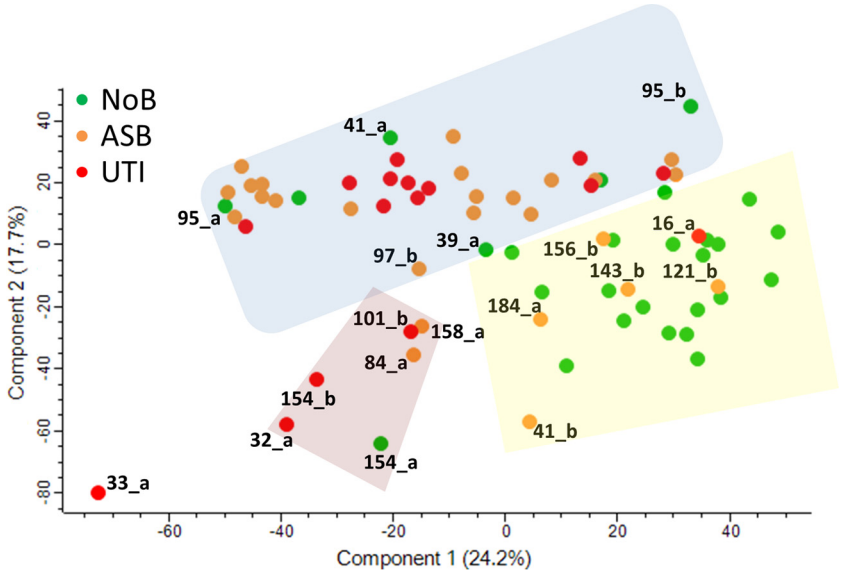

FIG 3 Principal component analysis shows similarities of a subset of ASB samples to NoB samples. All 69 proteomic profiles were subjected to PCA and sorted into three color-coded groups as indicated by the key in the figure. Numbered samples represent many ASB cases and a few NoB and UTI cases. The cases are discussed in the main text. PCA was performed on datasets limited to the overall 800 most abundant proteins, and results are shown in a two-dimensional space. We applied PCA to determine patterns of similarity and dissimilarity among the three groups. Two principal components that captured 41.9% of the total variance in the urine proteome are shown. LFQ values were subjected to $\log_2$ transformation; missing values were replaced using a data imputation program in Perseus software, version 1.5.0, with a default width of 0.3 and a downshift of 1.8. The imputations create a Gaussian distribution of random numbers based on the means and standard deviations of the measured values, simulating signals for proteins of low abundance (33). Shaded areas highlight clusters of samples with the following features: blue shading for high levels of neutrophil infiltration, usually in the presence of pathogens; red shading for high levels of hematuria with UTI and ASB cases; and yellow shading for low-level inflammation dominated by NoB cases but also with the inclusion of a few ASB cases.

malized data using the TPA method. In summary, there is no evidence of biological differences in the host immune responses to ASB versus UTI for the elderly cohorts under study here.

Following this, we assessed individual cases of ASB and UTI with low NAD scores. There were five ASB cases and four UTI cases with NAD scores of lower than 25% (Fig. 2). Only one ASB case, 121_b, had high quantities of KNG1 and increased quantities of ANGPTL2 and MXRA8 relative to the total proteome compared to UTI and ASB profiles with high NAD scores. PCA placed 121_b near most of the NoB cases, supporting the notion of urethral colonization in this patient. This analysis, displayed in a two-dimensional space in Fig. 3, represents a combination of the two components with the largest variation among the UP sample classes. The other ASB and UTI cases with low NAD scores were clustered either in the lower left red-shaded area of the plot in Fig. 3 and had evidence of hematuria or were clustered with 121_b and most NoB profiles in the center-right of the plot (yellow-shaded area). That cluster, composed of three additional ASB cases (184_a, 156_b, and 143_b) and one UTI case (16_a), had little evidence of hematuria. Based on the clustering results, there were only one UTI case and four ASB cases indicative of low-level immune responses in the absence of urothelial injury. This accounts for only 15% of the ASB cases. One ASB case had more than 10^5 bacterial cells per ml urine and an NAD score of less than 25%; this was 121_b, the case with increased KNG1, ANGPTL2, and MXRA8 quantities in the urinary pellet proteome. All other cases had 10^4 to 10^5 CFU per ml, indicating the possibility of an asymptomatic stage preceding UTI. The patients were not treated with antibiotic drugs, and it was unclear whether any UTI symptoms developed later.

DISCUSSION

As proposed by Stamm in 1983, bacteriuria reflects either urinary colonization without evidence of urothelial tissue invasion or UTI with clinical, histological, and immunological evidence of tissue injury and invasion, which is inferred from the measurement of pyuria. The presence of 10 leukocytes/mm^3 in uncentrifuged urine was defined as the nonpathological limit of pyuria (18). In an era of progressing antibiotic resistance and even of pan-resistance in microbial pathogens that are part of the ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (46), Nicolle et al. described guidelines for diagnosis and treatment of ASB and recommended that most cases of ASB where a considerable risk of complications is not obvious should no longer be treated with antibiotic drugs (9). The authors summarized differences in the frequency of ASB entailing pyruria for distinct patient cohorts, citing 30% to 70% and 90% of pyuria-associated cases in short-term-catheterized and elderly institutionalized patients, respectively. The number was 30% for young, otherwise healthy women. As stated in a review describing the challenges for UTI diagnosis and treatment, advanced methods could elucidate the role of the innate immune system in response to invading pathogens and distinguish bladder infection from renal infection with higher specificity (1).

Advanced proteomic analysis of clinical urine sediment samples was recently shown to characterize the human immune responses to uropathogens (29). Using that approach here, we examined the criteria that distinguish UTI from ASB on a molecular level and with statistical methods. The cohort consisted of 53 elderly patients hospitalized due to trauma and 100 urine samples that were surveyed to obtain insights into innate immunity, inflammatory responses, and urothelial injury. The differential clinical diagnosis of UTI versus ASB in this cohort had one confounding factor, the trauma suffered by these patients. The use of analgesics, distraction resulting from pain and shock, and temporary immune suppression could make it more difficult for a clinician to diagnose UTI. However, comorbidities, an altered sensation of pain, chronic pain, and ingestion of analgesics are issues affecting the elderly in general and make the diagnosis of UTI more difficult. As mentioned above, the frequency of pyuria combined with a diagnosis of ASB in the elderly is quite high. No matter whether patients examined for urinary tract symptoms have few or many comorbidities, standard criteria for diagnosis of UTI are applied widely, were applied here, and are relevant in the context of Medicare reimbursements. We excluded human subjects with evidence of other urogenital diseases from the study to avoid incorrect diagnoses of UTI and ASB.

Our proteomic surveys revealed similar intensities of antibacterial and inflammatory immune responses in the large majority of ASB and UTI cases. Importantly, they were clearly different from those in cases where bacteriuria was absent. Clustering of urinary proteome profiles suggested urethral colonization in only 15% of all ASB cases. Interestingly, urinalysis and proteomic evidence of vascular injury and hematuria, which is relatively common in elderly catheterized patients, did not confound the evidence that neutrophil-driven immune responses were equally prevalent in ASB and UTIs. Hematuria cannot be considered to be supporting clinical evidence of UTI or ASB in the catheterized patients under study here, a conclusion also mentioned in a review of the dipstick hematuria method (47). As recently reviewed in the context of common pathogens causing UTI in nursing home patients (5), we found that *Escherichia coli* was the most frequently identified pathogen, followed by *Enterococcus* sp. The two taxa were equally represented in the patient groups diagnosed with UTI and ASB in this study. We observed only a moderately increased prevalence of uropathogens adapted to a lifestyle on urethral catheters, such as *P. mirabilis*, *P. aeruginosa*, *Enterobacter*, *Citrobacter*, and *Candida* sp. (48). These species were identified in 10 of the 43 cases. While 19 cases met the definition of CAASB and CAUTI, most patients were catheterized for only a short period, and a single pathogen appeared to cause the bacteriuria in all but four cases.

The evidence was strong for the dominant role of neutrophils as the effector cells of the antibacterial and inflammatory response. In particular, proteins expressed in azurophilic granules of neutrophils (41) were highly abundant in proteomic datasets pertaining to the ASB and UTI cases, in contrast to the lower abundance seen in nearly all of the NoB cases. They have bacterial-surface-binding and enzymatic properties (49) or are implicated in the formation of granules, degranulation, cellular adhesion, and cytoskeletal rearrangements to enable phagocytosis (50). The latter protein category included the $\alpha M\text{-}\beta_2$ integrin complex (CD11b/CD18) and grancalcin (50), myosin-If (51), and the CD177 neutrophil surface marker (52). The myeloid cell nuclear differentiation antigen (MNDA) manipulates neutrophil apoptosis (53). Two previous studies identified neutrophil proteins via proteomics from urethral catheter samples but did not include experiments comparing ASB patients to UTI patients (13, 21). We conclude that fundamental defense mechanisms in elderly pa-

tients diagnosed with ASB implicate neutrophils as effector cells to kill the invading pathogen. Our findings enhance existing cellular evidence of pyuria in elderly ASB and CAASB patients (18).

Interrogation of pathways mediating UTI symptoms such as fever, pain, frequent urination, and dysuria was of interest. Prostaglandins and leukotrienes are eicosanoids synthesized from arachidonic acid via the activities of a set of well-characterized enzymes. Leukotrienes are produced by cells of the innate immune system, and LTB_4 is a potent neutrophil chemoattractant and stimulator of leukocyte adhesion to endothelial cells (54). LTD_4 is a potent eosinophil chemoattractant. As shown in Fig. 1, proteomic data on the abundance of enzymes that are part of the leukotriene synthesis and metabolism pathways were consistent with increased concentrations of LTB_4, while the decrease in the abundance of GGT1 suggested lower levels of LTD_4 in the urinary tract of patients who contracted ASB or UTI. We postulate that leukotrienes play a role in neutrophil chemotaxis in the urinary tract after pathogen recognition. COX2 is a key enzyme for the synthesis of prostaglandins and was reduced in abundance in comparisons of the ASB and UTI groups with the NoB group. Prostaglandins may act as pro- and anti-inflammatory signals and are not known as effectors of neutrophils (54). Although in-depth characterization of eicosanoids in the urinary tract is required to assess their roles in the sensation of pain and the occurrence of fever, our data support the notion that leukotrienes contribute to inflammation and pain signaling upon activation of the immune system in ASB and UTI cases, consistent with a study on neuropathy where leukotrienes were found to activate their nociceptive receptors (55). Our data also suggest that enzymes degrading leukotrienes, especially those facilitating β-oxidation (43), are less active; thus, LTB_4 levels are maintained as long as pathogens are present. Voltage-dependent anion channels (VDAC) reside in mitochondrial and plasma membranes of various cell types and play a central role in the regulation of the energy metabolism in neurons (56). VDAC1 and VDAC2 were decreased in abundance in the ASB datasets versus NoB datasets in the proteomic survey. Unlike voltage-gated sodium channels, VDACs have been implicated in the pathophysiology of neurotransmission pertaining to overactive bladder and dysuria (57). Another protein that was increased in abundance in the ASB and UTI proteomic profiles was synaptic vesicle membrane protein 1 (VAT-1), which was identified as a phosphatidic-acid-binding protein translocated to neutrophil membranes upon stimulation with N-formyl-methionyl-leucyl-phenylalanine (58). Interestingly, a VAT-1 ortholog in the marine ray (*Torpedo californica*) was described as a protein that is transported to cholinergic nerve terminals and abundant in synaptic vesicles. Its function in cholinergic neurotransmission has not been elucidated to date (59). Interestingly, overactive bladder was associated with hypersensitivity to cholinergic agonists in nerve endings of the urothelium (57). We hypothesize that VAT-1 is translocated to human neutrophil membranes and released into secretory vesicles and the synaptic space in bladder tissue nerve endings. This mechanism would then suggest that local neurological functions for fever and pain signaling are not perturbed in ASB patients.

The number of ASB cases in elderly trauma patients with low neutrophil protein content in urine, which supports the concept of bladder colonization (18), was low (at most four cases). We did not see a strong trend of lower bacterial cell counts per milliliter of urine for ASB compared to UTI cases, which would support the concept of emerging UTI in an asymptomatic stage. Five ASB and UTI cases with lower than 10^5 CFU/ml had proteomic evidence of neutrophil infiltration. To conclude the discussion, our data support the notion of activation of innate immune responses in UTI and ASB cases for elderly trauma patients. Treatment of ASB with antibiotics, especially in the hospital environment, has been discouraged due to the threat of emergence of multidrug resistances in typical uropathogens (3, 9). This is a sensible guideline given that complications such as preterm birth, pyelonephritis, and urosepsis are relatively rare in ASB patients. However, our data represent a step toward questioning the rationale of treating UTI and ASB in elderly patients differently with antibiotics. If the important aspect of UTI treatment is to alleviate symptoms, one could argue that anti-inflammatory and analgesic drugs should be administered. Therapy with antibiotic drugs could wait until symptoms persist for several days.

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The contents of the paper are solely our responsibility and do not necessarily represent the official view of NIH.

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